Protective Effect of Cocoa Extract on Albino Mice Skin Tumor After Exposure to 7,12 Dimethylbenz-$	ext{a}$ Antrasen (DMBA) and 12-O-Tetradecanoylphorbol-13-Acetate (TPA) Malondialdehyde (MDA) analysis and Histopathology

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ARTICLE INFO

Keywords:
Cocoa
DMBA
Histopathology
MDA
TPA

ABSTRACT

Aims. Oxidative stress result in DNA destruction in the induction cells. If this damage is irreversible, DNA will undergo mutation and may lead to cancer. One of cancer caused chemical and often use in experimental study is DMBA/TPA. Thus the chemical is utilized in this study to examine protective effect of cocoa extract on mice. Method. 30 albino mice, age 6-9 weeks, weight 20-30 gr were used as sample study. The animals were divided into 6 groups with different treatment: group 1, is negative control which receive no treatment; group II is control group receiving DMBA/TPA; group III receiving acetone, and group IV, V, and VI each receiving cocoa extract of 200, 400 and 800 ppm, respectively. Group II-IV expose to DMBA three times a week with every other day fashion in week 1, followed by application of 4μg TPA on week 2-12. All the animals were sacrificed 24 h after exposure and samples were taken and process for MDA and histopathology examination. Result. MDA level in 400 and 800 ppm cocoa extract groups were higher than control, while it is lower in 200 ppm group than in control. Anova result showed no significant differences among control and cocoa groups (p > 0.05). Histopathology examination result on DMBA/TPA control, acetone and 200 ppm cocoa groups consist of severe dysplasia and squamous cell carcinoma, whereas in 400 and 800 ppm cocoa groups some samples exhibit mild dysplasia. Kruskall Wallis statistical analysis indicates significant differences among control and cocoa groups (p ≤ 0.05). Conclusion. Topically applied 400 and 800 ppm cocoa exhibit protective effect on cancer formation with the best protection effect shown in concentration of 800 ppm.

1. Introduction

Cancer occurrences, including skin cancer, is a multiphase process. In vitro and epidemiology study indicate the carcinogenesis process occurs in several phases. Apoptotic and cell proliferation occur in different rates but still in balance condition throughout initiation and promotion phase. In progression phase the changing in balance condition enable cancer formation.

One of chemicals known to induce carcinogenesis phase is 7,12-dimethylbenz-$	ext{a}$ anthracene (DMBA) as initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as promoter. Initiator agent is genotoxic which contribute to the increase in skin cancer risk in human and experimental animal, thus it is often use to observe carcinogenesis process especially as mutagen prototype and modulator of signal transduction.

Exposure to chemical carcinogenic agent induce the formation of reactive oxygen species (ROS) which will provoke DNA, RNA and protein damage through chemical reaction such as oxidation, nitration, and halogenation. These events will increase mutation, altered protein and essential enzyme function. Several studies have indicate that ROS formation occur at initiation, promotion and progression phase through genotoxicity. Cell membrane is sensitive to ROS and become the target of destruction caused by ROS induction. Unsaturated fatty acids generally consist of methylene groups between two double bonds, thus make it more sensitive to oxidation. ROS react with fatty acid on cell membrane forming lipid peroxide, furthermore forming specific and carcinogenic malondialdehyde (MDA). This product is...
endogenous genotoxic agent made through enzymatic process and oxygen radicals induced by lipid peroxide, thus it can be used to evaluate DNA oxidative damage after DMBA exposure to the skin. Double-stranded DNA exposure to MDA modified its DNA and increase mutation rate if it replicates in mammalian cells. Destruction of DNA from the exposure indicate mutagenic nature of MDA in relation to oxidative stress and lipid peroxide. The result of this study indicates specific DNA destruction caused by oxidative stress is the formation of 8-OHdG. DMBA exposure to breast epithelial stem cell has proven the increasing oxidative damage on nuclear DNA (8-OHdG).

DMBA application onto skin as initiator indicates by specific mutation on an eukene. Furthermore, latent phase of tumor formation decrease significantly with application of TPA, thus both agents will form benign tumors (papilloma). However, the feasibility of papilloma formation in mice strain depends on carcinogen types, application doses and evaluation period. Study using 3 mg DMBA in 50 al acetone with 3 days interval followed by application of TPA 10 μg twice a week for 8-9 weeks result in skin tumor 34.9% from sample. Our previous study indicates DMBA exposure at 10 μg three times a week in the first week followed by application of 4 μg TPA three times a week in the second until week 10 on mice induce formation of papilloma and squamous cell carcinoma, thus enabling the use of the dose for the following study to induce tumor formation and protective effect trials on various natural ingredients which possess antioxidant and anti-proliferative effect.

Skin tumor formation after DMBA/TPA exposure is caused by DNA mutation, and alteration in gene regulates apoptotic balance, which play role in cell death if DNA damage is irreversible. Apoptosis occur in two main pathways, that is extrinsic or sitoplasmic pathway and intrinsic or mitocondrial pathway which enable the releasing of cytochrome C from mitochondria and apoptotic activation signal when the pathway is stimulated. Both pathways will induce caspase which later causes apoptosis. One of the most important regulator gene in the intrinsic pathway is BCL-2. It is first identified on translocation of chromosome 11 to 14 in follicular non-Hodgkin lymphoma, and as key regulator in apoptotic. Kedua jalur tersebut akan menginduksi caspase yang akhirnya menginduksi apoptosis. Apoptotic is controlled by gene that induce apoptotic (e.g. BAX) and anti-apoptotic gene (BCL-2). Overexpression of BCL-2 in the intrinsic pathway inhibit the extrinsic one. Various studies have proven that the increasing in BCL-2 expression which inhibit apoptotic play roles in the tumor formation induced by DMBA/TPA. Administration of arogenous extract indicative protective effect on tumor through induction of pro-apoptotic gene p53 and BAX, and inhibition of anti-apoptotic gene expression BCL-2.

Flavonoid is the greatest group of polyphenol which possess specific antioxidant nature beneficial for human healthiness. Various natural ingredients included in the group are green tea, fruits and vegetables has biologic effect on proliferation and angiogenesis and decreasing cardiovascular and cancer risk. Other natural ingredient with strong antioxidant activities is cocoa. Cocoa extract has some active antioxidant ingredients including flavonoids, catechins, epicatechins dan proanthocyanidins. Ex vivo and in vivo studies indicates that epicatechin is the major polyphenol in cocoa and cocoa extract, and exhibit inhibitory effect on lipid peroxide plasma because of its ability to bind on low density lipoprotein. Stress oxidative induction with H2O2 induce BCL-2 downregulation result in apoptotic. Cocoa procyanidin as antioxidant has proven inhibit apoptotic through inhibition of downregulation BCL-2 expression on tissue induced with H2O2. On the contrary, cocoa rich diet has proven inhibit tumor formation at the early stage of colin carcinoma on experimental animal. This inhibition effect attained through antioxidant effect, and increasing of endogenous enzymatic and nonenzymatic defence. Cocoa also exhibit anti-proliferative effect through decreasing extracellular regulated kinases, kinase protein and dan cyclin D1 together with prosapoptotic effect as indicated with decreasing Bcl-xL levels and increasing level of Bax dan caspase-3 activities.

Despite the already established effect of cocoa as antioxidant on various diseases, protective effect of various topical application concentration on carcinogenesis process as antioxidant has not so far been established, especially protective effect of cocoa extract on skin tumor formation by evaluating MDA. Thus, further study are needed on the effect of topical cocoa seed extract on mice receiving various concentrations of cocoa extract and DMBA/TPA application.

MATERIAL AND METHODS

Study design. This study is a true experiment post design with control group. It was conducted at Animal laboratory of Medical Faculty of Hasanuddin University (Mataram, Indonesia). Histopathology preparation was made at the Pathology anatomy and biotechnology laboratory of Hasanuddin University's Hospital.

Study subject. The subject of this study were 6-9 weeks old albinio mice from one mother purchased from Balithang veterinary center in Maros, South Sulawesi. The mice were housed in standard condition for 1 week; room temperature (28 ± 2°C), humidity 50 ± 10% and subjected to a 12 h light/12 h dark cycle.

Materials and Equipments. Cocoa extract with concentration of 200, 400 and 800 PPM in lotion preparation, 50 μg 7,12-Dimethylnbenz (J) Anthracene (DMBA), 4 μg 12-O-Tetradeutocanophybor-13-Assecat (TPA) and DP12 type of olympus microscope were used.

Study sample. The sample of this study consist of 30 mice which were divided into several perpetuation groups. Inclusion criteria were healthy female albinio mice species swiss albinio mice aged 6-9 weeks old, weighing 20-30 g. Mice which became sick or dead throughout the study were excluded.

Methods. Sixty mice were divided into 6 groups, group 1 were exposed to DMBA/TPA (10 mice), group 2 were treated topically with lotio base (control) together with exposure of DMBA/TPA (15
mice), group III-V each consisted of 10 mice were treated topically with 200, 400, and 800 PPM cocoa extract, respectively, 20 minutes before exposure to DMBA/TPA. DMBA/TPA were exposed in an everyday fashion for 12 weeks. The mice were terminated 24 hours after the last exposure and examined for MDA level and histopathologic feature.

Intervention and outcome measurements. Documentation were obtained from all the mice included in the study for initial data and throughout the study. The mice were acclimatized 1 week before use. They were randomly put in groups according to each treatment. DMBA/TPA and cocoa extract were applied onto shaved interscapular areas. The perpetration were done for 12 weeks, and at the end of week 10 all the mice were sacrificed. Excisional biopsies with 2 cm diameter were taken for MDA and histopathologic examination. For histopathology, the sample were taken from mice’s ear, fixe in formaline buffer 10%, and cut in blocks with 4-5 μm thickness. They were deparaffinated with xylene and stained with hematoxylin eosin (HE) for standard evaluation using olympus CV microscope. MDA were calculated using Wright methods. The last concentration were calculated using molar coefficient of 1.56×105/M/cm. Lipid peroxide inhibition determined by measure the decreasing number of MDA in the incubation compare to acetone control.

RESULTS

Sample characteristics. This study was performed on 30 albino mice eligible for inclusion criteria weighing 20-30 g (20.97 ± 2.680) randomly divided into groups of each treatment. Natural ingredients used in this experiments were already tested for the flavonoid content, with concentration of 100, 200, 400, and 800 ppm of cocoa extract contain 7.42; 14.48; 29.68; and 59.36 μg of total flavonoid content respectively.

MDA level: in every treatment groups expose to DMBA/TPA and cocoa extract.

Data from table 1 shows no significant differences among the treatment groups (p>0.05). Nevertheless, there are increasing level of MDA in treatment group with topically applied 400 and 800 PPM cocoa compare to DMBA/TPA control and acetone. All treatment groups had a higher MDA level than the negative control. Graph 1 shows the feature of MDA level on study sample.

Histopathologic feature of skin tissues of every treatment groups expose to DMBA/TPA and cocoa extract.

Data from table 2 shown frequency of distribution from sample after DMBA/TPA exposure for 12 weeks. In the control group without protection (DMBA/TPA control and DMBA/TPA + acetone control) and group of 200 ppm cocoa extract, severe dysplasia and squamous cell carcinoma occur; while in 400 and 800 ppm of cocoa extract, mild dysplasia occur in 20% and 40% of samples, respectively. Statistical analysis result indicates significant differences among treatment groups p<0.05. The result of histopathologic feature as shown in figure 1–5.

### Table I. Level of tissue MDA after exposure with DMBA/TPA for 12 weeks according to cocoa concentration in acetone solution groups as protector

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean ±SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA/TPA control</td>
<td>3.67±0.598</td>
<td>0.386*</td>
</tr>
<tr>
<td>Acetone control</td>
<td>2.31±3.252</td>
<td></td>
</tr>
<tr>
<td>Cocoa 200</td>
<td>2.14±0.668</td>
<td></td>
</tr>
<tr>
<td>Cocoa 400</td>
<td>4.37±3.194</td>
<td></td>
</tr>
<tr>
<td>Cocoa 800</td>
<td>4.39±4.028</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>1.16±1.338</td>
<td></td>
</tr>
</tbody>
</table>

*Anova statistical analysis

### Table II. Frequency of histopathologic feature distribution according to treatment

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Hyperplasia</th>
<th>Mild Dysplasia</th>
<th>Severe Dysplasia</th>
<th>Squamous Cell Ca.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA/TPA control</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>DMBA/TPA + Acetone</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Aceottine + Cocoa 200</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Aceottine + Cocoa 400</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Aceottine + Cocoa 800</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>30</td>
<td>50</td>
</tr>
</tbody>
</table>

### TableIII. Mice group expose to DMBA and TPA for 12 weeks

*Kruskal Wallis statistical analysis

Mice group expose to DMBA for 7 days and TPA for 11 weeks

Graph 1. Level of MDA tissue expose to DMBA/TPA for 12 weeks based on conca concentration in acetone solution as protector
Graphic II: The effect of cocoa extract on mice exposure to DMBA/TPA for 12 weeks based on histopathologic feature

Figure 1. Normal skin tissue, Cell dysplasia was not found, cell polarity is still good

Figure 2. Mild dysplasia at less than 1/3 of the epidermis

Figure 3. Moderate dysplasia at 1/3 - 2/3 of the epidermis

Figure 4. Severe dysplasia at more than 2/3 the epidermis of the epidermis

Figure 5. Squamous cell carcinoma, showed expansion of atypical keratinocytes that pass through the basal membrane and into the dermis to form clumps of cells were detached from the epidermis
Discussion

Oxidant role in skin cancer development is a complex aging process, comprising of three phases: initiation, promotion and progression, which is mediated by various alteration in molecular, cellular and biochemical. ROS is involved in these three phases. ROS is generally found in cell membrane as malondialdehyde, product of enzymatic process and lipid peroxide oxidation. Various studies indicates that MDA induce mutation that result in cancer. This study utilize MDA level measurements to evaluate the extent of oxidative stress induced by DMBA exposure to mice. ELISA result indicates that DMBA induce oxidative stress in DMBA/TPA control group with higher level than negative control. Whereas in Arova statistical analysis, MDA level in coca group is not statistically significant with negative control. Despite this result, there is tendencies of higher level in coca group compare to DMBA/TPA control which indicates the effect of coca extract on stress oxidative induction. This result is different to other studies which indicates that coca may inhibit stress oxidative formation by heat exposure on leukocyte winstran. The differences may be caused by cell target on this study is the cell which is already undergo transformation resulting from DMBA/TPA induction. On in vitro and in vivo studies, exposure of coca polyphenol extract with concentration 10-160 μL on RLE cell may substantiate hydrogen peroxide induction on oxidative stress accumulation. Other study on cancer cells indicates increasing oxidative stress on cancer cell induced by nitrogen-activated protein kinases (MAPKs) inhibitor which enables prematur senescence. Thus, protective effect of coca extract may occur through apoptotic and or premature senescence pathways.

DMBA induction in initiation phase, followed by TPA application as promoter agent result in histopathologic alteration as severe dysplasia and squamous cell carcinoma compare to unexposed mice skin. Several experiment indicate that diet rich in coca possess antiproliferative effect on winstara rat colon cancer induced by azoxymethane. Coca procyanidin and E2 procyanidin in concentration of 5μg/mL and 40μM, respectively. Inhibit induction of neoplastic cell (BJ6P) by TPA as high as 47% and 93% through suppression on kinase activity. Coca also inhibit COX-2, AP1 and NF-KB expression related to cancer. Antimutagenic effect of coca extract has already proven in vitro studies using benzo[a]pyrene as mutagen, which indicate coca inhibition on cancer initiation through metabolic activation inhibition by cytochrom p450 (CYP), typically CYP1. In the histopathologic graphic, it is shown that 200 ppm coca group has similarity in relation to cancer occurrence with control group which exhibit severe dysplasia and squamous cell carcinoma, thus it is ineffective as cancer protector in the promotion/progression phase in carcinogenesis study. Topically applied 400 and 800 ppm coca group exhibit inhibition effect on skin tumor. In these concentration, some sample still exhibit mild dysplasia. It is as shown in the result of Kruskall Wallis statistical trials which is significantly different with control group (p=0.05) with concentration of 800 ppm as the best protective concentration. In association with MDA level in this concentration, there maybe a tendency that the increasing MDA level has a role in coca protection effect, evenmore in comparison to 200 ppm coca which has lower MDA level. Increasing level of MDA probably induce apoptotic and or senescence pathway. Further study in association with both pathways will be required.

CONCLUSIONS

Topically applied 400 and 800 ppm coca exhibit protective effect on cancer formation with the best protection effect shown in concentration of 800 ppm.

ACKNOWLEDGEMENT

We thank the laboratory of Pharmacognosis and Phytochemistry of Pharmacy Faculty and Microbiology Laboratory of Medical Faculty of Hasanuddin University for the support.

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